Molecular Analysis of Phyllodes Tumors Reveals Distinct Changes in the Epithelial and Stromal Components

Elinor J. Sawyer,^{*†‡} Andrew M. Hanby,[†]
Paul Ellis,[‡] Sunil R. Lakhani,[§] Ian O. Ellis,[¶]
Sue Boyle,^{||} and Ian P. M. Tomlinson^{*†}

From the Molecular and Population Genetics Laboratory,^{*} Imperial Cancer Research Fund, London; the Hedley Atkins/Imperial Cancer Research Fund Breast Pathology Laboratory,[†] Guy's Hospital, London; the Guy's, King's, St. Thomas's Cancer Centre,[‡] St. Thomas's Hospital, London; the Department of Histopathology,[§] Royal Free & University College Medical School, London; the Department of Histopathology,[¶] City Hospital, Nottingham; and the Department of Histopathology,^{||} Northwick Park Hospital, Harrow, United Kingdom

Phyllodes tumors are fibroepithelial mammary lesions that tend to behave in a benign fashion but may undergo sarcomatous transformation. A study of clonality in these tumors has suggested that the epithelial component is polyclonal, but the stroma is monoclonal, and thus forms the neoplastic component of the lesion. In this study microsatellites on chromosome 1q and chromosome 3p were assessed for allelic imbalance (AI) in 47 phyllodes tumors; in all cases stroma and epithelium were analyzed separately. Ten of 42 (24%) phyllodes tumors showed AI at one or more markers on 3p, and 14 of 46 (30%) showed AI on chromosome 1. Five tumors had changes in both the epithelium and stroma. Eight tumors had changes only detectable in the stroma and eight, changes in the epithelium only. Three tumors exhibited low-level microsatellite instability in the epithelium but not in the stroma. The results show that AI on 3p and 1q does occur in phyllodes tumors and that it can occur in both the stroma and epithelium, sometimes as independent genetic events. These unexpected findings throw into doubt the classical view that phyllodes tumors are simply stromal neoplasms and raise questions about the nature of stromal and epithelial interactions in these tumors. (*Am J Pathol* 2000, 156:1093-1098)

Phyllodes tumors are fibroepithelial neoplasms of the breast, accounting for about 0.5% of all breast tumors.¹ They are composed of stromal and epithelial elements analogous to fibroadenoma, although the stromal element is more cellular and may outgrow the epithelium.

Clinically most of these tumors tend to behave in a benign fashion, but, unlike fibroadenomas, they can recur locally and can undergo malignant progression to sarcoma.² Histologically, phyllodes tumors are classified as benign, borderline, or malignant on the basis of stromal cellularity, nuclear atypia, mitotic activity, stromal overgrowth, and type of border (infiltrating or pushing). There are limitations to this classification, as it does not always correlate with clinical outcome.³ In view of the progression to sarcoma in some cases, the stroma of phyllodes tumors is generally thought to be the neoplastic element. Clonality studies of five tumors have suggested that the stroma is monoclonal and the epithelium polyclonal.⁴ Hyperplastic epithelium may, however, be found in some phyllodes tumors and there is reported to be an association with lobular carcinoma *in situ* (LCIS) and infiltrating ductal carcinoma of the breast.⁵

There have been few molecular studies of phyllodes tumors. Comparative genomic hybridization (CGH) analysis of 18 whole, fresh-frozen phyllodes tumors revealed that loss of material on 3p and gain of 1q were the two most common chromosomal abnormalities.⁶ These changes are also common in breast carcinoma.^{7,8} These observations suggest that the same genes are involved in the pathogenesis of both diseases. However, the possibility exists that phyllodes tumors might be less genetically heterogeneous and thus more amenable to gene mapping studies. We have used microsatellites on chromosomes 1q and 3p for assessment of allelic imbalance (AI) in 47 phyllodes tumors to confirm the CGH findings and to provide finer-scale mapping of the regions of loss and gain. In all tumors, stroma and epithelium were microdissected and analyzed separately. We have also used the same set of microsatellites on an unselected set of 78 breast carcinomas to see if similar areas of loss and gain occur in both breast and phyllodes tumors.

E. J. S. is supported by the Special Trustees of Guy's & St. Thomas's Hospital.

Accepted for publication November 17, 1999.

Address reprint requests to Dr. E. J. Sawyer, Molecular and Population Genetics Laboratory, Imperial Cancer Research Fund, 44, Lincoln's Inn Fields, London WC2A 3PX, UK.

Table 1. Histological Classification of Phyllodes Tumors

Sample	M	SC	SA	EA	SM	EM	EH	SO
52	0	1	2	0	2	1	2	2
53	2	2	2	0	2	1	1	1
54	1	1	1	0	0	0	0	0
55	1	2	2	1	3	1	0	2
56	1	1	2	1	2	1	2	1
57	2	1	1	1	3	1	0	2
58	0	0	1	1	0	0	0	0
59	0	0	1	1	0	0	0	0
60	0	2	2	1	3	0	0	0
61	0	1	2	0	2	1	2	2
62	0	0	0	0	2	1	0	0
63	0	0	0	0	0	1	0	0
64	0	0	0	0	0	0	0	0
65	1	1	0	1	0	0	2	2
66	1	0	0	2	0	2	2	0
67	0	0	0	1	0	0	1	1
68	2	1	1	1	1	1	0	1
69	0	0	0	1	0	0	0	0
70	0	0	1	2	0	0	0	1
71	0	0	0	0	0	0	1	1
72	0	0	0	0	0	0	0	0
73	0	0	0	2	0	2	1	0
74	2	0	1	1	0	1	2	1
75	2	1	1	1	0	0	0	2
76	0	0	0	0	0	0	0	0
77	2	2	1	2	2	2	1	1
78	2	0	0	0	0	1	1	0
79	0	2	2	0	2	2	2	2
80	0	1	1	1	2	3	1	1
81	2	1	0	0	1	0	1	1
82	1	1	1	0	1	1	1	0
83	1	1	1	0	1	2	2	1
84	1	2	1	2	1	0	0	0
85	0	0	1	2	0	0	1	1
86	0	0	0	0	0	0	1	0
87	0	1	0	1	0	0	1	0
88	0	1	0	0	0	0	1	1
89	1	1	1	0	1	0	0	1
90	1	0	1	0	0	0	0	2
91	2	0	0	0	0	0	1	1
92	0	1	0	0	0	0	0	0
93	0	0	0	0	0	0	2	0
94	0	1	0	0	1	2	1	0
95	2	2	1	2	1	2	1	2
96	2	2	2	2	2	2	1	2
97	1	1	2	2	1	0	2	2
98	2	2	2	0	3	0	1	1

Margins (M): 0, crisp; 1, blurred/focally infiltrative; 2, widely infiltrative. Epithelial/stromal atypia (EA; SA): 0, none; 1, some; 2, marked. Epithelial/stromal mitosis (EM/SM): 0, none; 1, found after hard search; 2, easily found; 3, numerous. Stromal overgrowth (SO) and epithelial hyperplasia (EH): 0, none; 1, slight; 2, marked. Stromal cellularity (SC): 0, average; 1, increased; 2, very cellular.

Materials and Methods

Forty-seven phyllodes tumor samples were collected from five centers. Hematoxylin and eosin (H&E)-stained slides of each case were reviewed by a single histopathologist (A. H.) to confirm the diagnosis and provide a consistent histological classification. The tumors were scored for stromal cellularity, stromal and epithelial atypia, stromal and epithelial mitoses, presence of epithelial hyperplasia, stromal overgrowth, and type of margin (see Table 1). All tumor material was archival paraffin-embedded tissue. Five sections (10 μ m) were cut from each tumor and stained with toluidine blue. Each section

Figure 1. **a:** Example of a phyllodes tumor showing leaf like stromal projections surrounded by epithelium. **b:** Microdissection of epithelium using the laser capture microscope from the same section. **c:** Dissected epithelium.

was microdissected to remove the epithelium, normal tissue, and stroma separately. The intratumoral epithelium was initially dissected using a laser capture microscope (Arcturus Engineering, Mountain View, CA; Figure 1). The stroma and surrounding normal tissue were then microdissected from each other by hand using a 25-gauge needle. The adjacent normal tissue was used as a source of constitutional DNA.

Extraction of DNA from the tumor and normal tissue was performed using the Qiagen (Hilden, Germany) Tissue Extraction Kit if there was more than 2 cm² of tissue. For smaller amounts of tissue, generally the epithelium, DNA extraction was performed by incubating at 55°C in 1× polymerase chain reaction (PCR) buffer (Promega, Madison, WI) at pH 8 with proteinase K at 200 µg/ml overnight.

Seventy-eight unselected, consecutive, fresh-frozen paired breast carcinoma and normal DNA samples were taken from the Guy's Hospital tumor bank for analysis.

For the initial mapping of 3p and 1q, 10 highly polymorphic microsatellite markers (5 for 3p, 4 for 1q, and 1 close to 1cen) were chosen from public databases (Genethon, www.genethon.fr, and CHLC, www.chlc.org). One oligonucleotide for these markers was fluorescently labeled (FAM, TET, or HEX) and product sizes were chosen in such a way that all 10 microsatellites could be run together in a single lane on the ABI 377 sequencer. Microsatellite loci were amplified in the PCR from stroma, epithelium, and normal tissue (phyllodes tumors) and from tumor and normal DNA (breast carcinomas). For each oligonucleotide pair, the PCR was optimized for annealing temperature and Mg²⁺ concentration. PCR was performed in a 25-µl volume. The PCR reaction typically contained 20–100 ng DNA, 50 mmol/L KCl, 0.5–2.5 mmol/L MgCl₂, 10 mmol/L Tris-HCl, 0.1% Triton, 2.5 µg of bovine serum albumin, 0.2 mmol/L of each dNTPs, 10 pmoles of each oligonucleotide, and 1.25 U *Taq* DNA polymerase (Promega, Southampton, UK). The PCR reaction consisted of an initial step of 94°C for 4 minutes, then 40 cycles of 1 minute at 94°C, 1 minute at the appropriate annealing temperature, and 1 minute at 72°C in a PTC-225 Peltier thermal cycler (MJ Research, Waltham, MA).

Microsatellites were analyzed for allelic imbalance (AI) using the Genotyper program (ABI). From the published CGH results, we assumed that all AI on 3p resulted from loss of material (or alternatives such as mitotic recombination), and that all AI on 1q reflected true gain of material. On 3p, AI at each marker locus was considered to be present if the area under one allelic peak in the tumor was less than 0.5× or greater than 2× that of the other allele, after correcting for the relative allelic sizes using the constitutional DNA. For 1q, less stringent thresholds (allelic ratios of less than 0.57 and greater than 1.75) were used, reflecting the expected gain of material, which might be of a low level.

The χ^2 test was used to look for an association between epithelial and stromal AI in phyllodes tumors and the histological parameters recorded.

Results

The histological features of the phyllodes tumors are detailed in Table 1. The majority were benign. Only two had frankly malignant stroma (samples 55 and 79). Forty-nine percent had an infiltrating border and 57% showed evidence of stromal overgrowth. There was a high incidence of epithelial hyperplasia within the phyllodes tu-

mors; 60% had some evidence of epithelial hyperplasia, and in 21% this was marked. One tumor contained a focus of LCIS (sample 95) in the epithelium and one had foci of both ductal carcinoma *in situ* (DCIS) and LCIS (sample 56).

Fourteen of 46 (30%) phyllodes tumors showed AI at one or more markers on 1q and 10 of 42 (24%) showed AI on 3p (Figures 2 and 3). Five tumors were not informative at any locus on 3p and one tumor was not informative at any locus on 1q. The breast carcinomas showed higher rates of AI, 40% (30/75) and 67% (50/75) on 3p and 1q, respectively (Table 2). All 10 microsatellites amplified consistently for the breast carcinomas, but only 7 amplified consistently in the phyllodes tumors, owing to the fragmented DNA extracted from the paraffin-embedded tissues. When just the 7 markers used for the phyllodes tumors were analyzed, 27/74 (36%) and 38/73 (52%) of the carcinomas showed AI on 3p and 1q, respectively.

In the phyllodes tumors, AI was not confined to the stroma. Some tumors had AI in the stroma only, but others had AI affecting both the epithelium and the stroma, or AI detectable only in the epithelium (Figures 2 and 3). In all cases, these changes were consistently detected in repeat PCRs from the same samples. Overall, allelic imbalance was slightly more common in the epithelium than in the stroma (Table 2). Looking at the group as a whole, 5 tumors had changes in both the epithelium and stroma, 8 tumors had changes only detectable in the stroma and 8, changes in the epithelium only. However, due to the small amount of tissue in the epithelial components, many more of these failed to amplify markers compared to the stroma. For the 8 cases where only the stroma showed AI, in 4 the corresponding epithelium would not amplify and thus it is not possible to exclude similar changes in the epithelium. For the remaining 4 tumors, however, the AI was confined to the stroma with no evidence of AI in the epithelium. One of these 4 tumors showed stroma-specific AI at markers on 1q and three, imbalance on 3p. In all 8 cases that showed AI in the epithelium only, the stroma had also amplified; thus these changes were definitely confined to the epithelium. Four of these 8 tumors showed epithelium-specific AI on 1q and three, imbalance on 3p; one tumor (sample 69) showed AI on both arms. Of the tumors with AI in both stroma and epithelium, 4 were entirely concordant (showing imbalance of the same allele at the same marker). In one tumor (sample 95), however, a complex pattern of AI was seen (Figures 2 and 3). Examples of AI in the stroma and epithelium are shown in Figure 4.

In addition to the AI detected, 3/47 (6%) phyllodes tumors exhibited microsatellite instability (MSI) in the epithelium (Figure 5). No MSI was demonstrated in the stroma. Again, these changes were consistently demonstrated in repeat PCR reactions. Only 2/78 (2.7%) breast carcinomas showed evidence of MSI.

The overall lower frequency of AI in the phyllodes tumors and the failure of some samples to amplify in the PCR mean that it was not possible to map accurately regions of allelic imbalance and a detailed comparison with the breast carcinomas was difficult. Nevertheless, there appeared to be some concordance between the

Figure 2. Map of AI on chromosome 1q in phyllodes tumors. Only tumors showing AI or MSI are shown.

patterns of AI on 1q in the two types of lesion (Table 2): the highest frequency of AI occurred toward the 1q telomere in the stroma and epithelium of the phyllodes tumors and in the breast cancers. On 3p, no obvious concordance existed. AI occurred at similar frequencies

along the 3p chromosome arm in the carcinomas, whereas maximum imbalance was at D3S1300 in the stroma of the phyllodes tumors and at D3S1293 in the epithelium (Table 2). This difference on 3p may reflect our original hypothesis that mapping losses and gains would,

Figure 3. Map of AI on chromosome 3p in phyllodes tumors. Only tumors showing AI or MSI are shown.

Table 2. Comparison of AI in Phyllodes Tumors and Breast Carcinomas

Marker	Phyllodes		Carcinomas AI/Informative cases (%)
	Stroma AI/ Informative cases (%)	Epithelium AI/Informative cases (%)	
D3S1297	—	—	8/59 (14)
D3S1293	1/21 (5)	3/11 (27)	14/57 (25)
D3S1619	1/31 (3)	0/18 (0)	9/51 (18)
D3S1581	—	—	11/56 (20)
D3S1300	3/25 (12)	3/21 (14)	10/57 (18)
D1S187	3/39 (8)	5/36 (14)	8/44 (18)
SPTA1	1/16 (6)	0/4 (0)	6/33 (18)
D1S416	2/36 (6)	3/30 (10)	13/58 (22)
D1S549	3/24 (13)	2/13 (15)	20/47 (43)
D1S1609	—	—	20/51 (39)

in theory, be easier in phyllodes tumors than in genetically heterogeneous carcinomas. Given our findings in the stromal and epithelial components of phyllodes tumors, however, such studies are likely to be problematic at this stage.

There was no significant association between AI in either the epithelium or stroma and the recorded histological variables (χ^2 test, details not shown). In particular, there was no association between AI at any marker and

Figure 4. Examples of AI (arrows) in (a) the stroma (sample 90, marker D1S416) and (b) the epithelium (sample 69, marker D1S187).

Figure 5. Example of microsatellite instability (arrows) in the epithelium. **a:** Sample 83, marker D3S1619. **b:** Sample 69, marker D3S1619.

epithelial hyperplasia or stromal overgrowth. The tumor with LCIS did show allelic imbalance at markers on both 1q and 3p, although the tumor with epithelium containing DCIS/LCIS showed no allelic imbalance.

Discussion

The general belief regarding phyllodes tumors is that the stroma is the neoplastic element and the epithelium within the tumors is entirely innocent, having either been caught up in the neoplastic stromal proliferation or shown some proliferation driven by stromally derived growth factors. With the use of the laser capture microscope, we have separated the stroma and epithelium with minimal contamination and studied each separately. This study has shown that AI in the epithelium of phyllodes tumors is as common as it is in the stroma. Thus, we suspect that, at least in some phyllodes tumors, both stroma and epithelium are neoplastic. Although contrary to general belief about the cellular origins of phyllodes tumors, our conclusions are supported by a previous cytogenetic analysis of stroma and epithelium in a small number of phyllodes tumors that showed chromosomal abnormalities in both tumor components.⁹

Our data also show that the genetic changes in the stroma and epithelium of individual phyllodes tumors are sometimes discordant. Thus, either both parts of the tumor have independent clonal origins, or the stroma and epithelium originate from the same clone and acquire different mutations during tumor progression. Theoretical considerations, namely the low probability of independent mutations in adjacent normal stroma and epithelium, and the finding of AI concordance in some phyllodes tumors suggest the latter. The finding of MSI in the epithelium of some phyllodes tumors is interesting in this regard and has not been reported before in benign breast disease. All examples of MSI in our phyllodes tumors were of the low-level type, which is believed not usually to be associated with mismatch repair deficiency. Some studies have found MSI to accompany early transformation of breast epithelium,¹⁰ and it is possible that the MSI we have detected is an epiphenomenal event associated with abnormal cell division of the epithelium in phyllodes tumors.

Allelic loss and cytogenetic rearrangements have been described on 3p in benign breast conditions such as papillomas, atypical epithelial hyperplasia, and fibroadenoma, as well as in phyllodes tumors and breast carcinoma.^{8,9,11} One of the most commonly deleted regions, in benign breast tissue as well as carcinoma, is 3p12-p14, reportedly including the *FHIT* gene.¹² D3S1300 maps to an intron in *FHIT*.^{13,14} Losses at both 3p14 and 3p21-p23 have been described in phyllodes tumors using cytogenetic methods.⁹ In our study, 3p AI at these sites occurred in both the epithelium and stroma. Gain of 1q is a common cytogenetic finding in many cancers. It was of particular interest in phyllodes tumors, as in a set of 18 tumors studied by CGH it appeared to be a marker of recurrence.⁶ In that study, 38% of tumors showed gain of 1q, compared to 30% with AI in our series. This difference may have resulted from the selection of fresh-frozen tumors for the previous study, which may have been larger and therefore more aggressive, or the different techniques, CGH and microsatellites, used to assess gain and loss.

The lack of correlation between the AI findings and histological observations is interesting. It may arise because 1q and 3p changes can occur relatively early in the growth of phyllodes tumors and other loci are involved in phyllodes tumor progression. The absence of a correlation between epithelial hyperplasia or atypia and epithelial AI is, in retrospect, surprising, but any correlation may be masked by a tendency for lesions without such epithelial changes to be more difficult to amplify in the PCR. This same tendency may mean that our reported frequencies of AI overestimate the true frequencies, especially in the epithelium.

The genetic changes we have described in both the epithelium and stroma of phyllodes tumors suggest that both are part of the neoplastic process. A recent study showed that the more malignant phyllodes tumors showed focal areas of increased stromal cellularity, often condensed around epithelial components, which corre-

sponded to areas of p53 immunoreactivity.¹⁵ Further studies must re-address the issue of clonal origins in early phyllodes tumors using new techniques. They must also analyze the dependence of stromal growth on the epithelium or *vice versa*, the stage (if any) at which independent growth of stroma and/or epithelium occurs, and the events which determine that transition.

References

1. Reynolds J, Mies C, Daly JM: Mesenchymal infiltrating tumors. The Breast: Comprehensive Management of Benign and Malignant Disease. Edited by Bland KL, Copeland. Philadelphia, Saunders, 1991, pp 210-221
2. Salvadori B, Cusumano F, Del Bo R, Delledonne V, Grassi M, Rovini D, Saccoczi R, Andreola S, Clemente C: Surgical treatment of phyllodes tumours of the breast. *Cancer* 1989, 69:2532-2536
3. Norris HJ, Taylor HN: Relationship of histological features to behaviour of cystosarcoma phyllodes: analysis of ninety four cases. *Cancer* 1967, 20:2090-2099
4. Noguchi S, Motomura K, Inaji H, Imaoka S, Koyama H: Clonal analysis of fibroadenoma and phyllodes tumour of the breast. *Cancer Res* 1993, 53:4071-4074
5. Christensen L, Nielsen M, Madsen PM: Cystosarcoma Phyllodes: a review of 19 cases with emphasis on the occurrence of associated breast carcinoma. *Acta Path Microbiol Immunol Scand Sect A* 1986, 94:35-41
6. Lu Y-J, Birdsall S, Osin P, Gusterson B, Shipley J: Phyllodes tumours of the breast analysed by comparative genomic hybridization and association of increased 1q copy number with stromal overgrowth and recurrence. *Genes Chromosomes Cancer* 1997, 20:275-281
7. Tirkkonen M, Tanner M, Karhu R, Kallioniemi A, Isola J, Kallioniemi OP: Molecular cytogenetics of primary breast cancer by CGH. *Genes Chromosomes Cancer* 1998, 21:177-184
8. Pandis N, Bardi G, Mitelman F, Heim S: Deletion of the short arm of chromosome 3 in breast tumours. *Genes Chromosomes Cancer* 1997, 18:241-245
9. Dietrich CU, Pandis N, Rizou H, Petersson C, Bardi G, Qvist H, Apostolikas N, Bohler PJ, Andersen JA, Idvall I, Mitelman F, Heim S: Cytogenetic findings in phyllodes tumours of the breast: Karyotypic Complexity differentiates between malignant and benign tumours. *Hum Pathol* 1997, 28:1370-1382
10. Huang Y, Bove B, Wu Y, Russo IH, Yang X, Zekri A, Russo J: Microsatellite instability during the immortalization and transformation of human breast epithelial cells in vitro. *Mol Carcinog* 1999, 24:118-127
11. Dietrich CU, Pandis N, Teixeira MR, Bardi G, Gerdes AM, Andersen JA, Heim S: Chromosome abnormalities in benign hyperproliferative disorders of epithelial, and stromal breast tissue. *Int J Cancer* 1995, 60:49-53
12. Matsumoto S, Kasumi F, Sakamoto G, Onda M, Nakamura Y, Emi M: Detailed deletion mapping of chromosome arm 3p in breast cancers: a 2cM region on 3p14.3-21.1 and a 5cM region on 3p24.3-25.1 commonly deleted in tumours. *Genes Chromosomes Cancer* 1997, 20:268-274
13. Man S, Ellis IO, Sibbering M, Blamey RW, Brook JD: High levels of allele loss at the FHIT and ATM genes in non-comedo ductal carcinoma in situ and grade 1 tubular invasive breast cancers. *Cancer Res* 1996, 56:5484-5489
14. Ohta M, Inoue H, Cotticelli MG, Kastury K, Baffa R, Palazzo J, Siprashvili Z, Mori M, McCue P, Druck T, Croce CM, Huebner K: The FHIT gene spanning the chromosome 3p14.2 fragile site, and renal carcinoma associated t(3;8) breakpoint, is abnormal in digestive tract cancers. *Cell* 1996, 84:587-597
15. Millar EKA, Beretov J, Marr P, Sarris M, Clarke RA, Kearsley JH, Lee CS: Malignant phyllodes tumours of the breast display increased stromal p53 protein expression. *Histopathology* 1999, 34:491-496